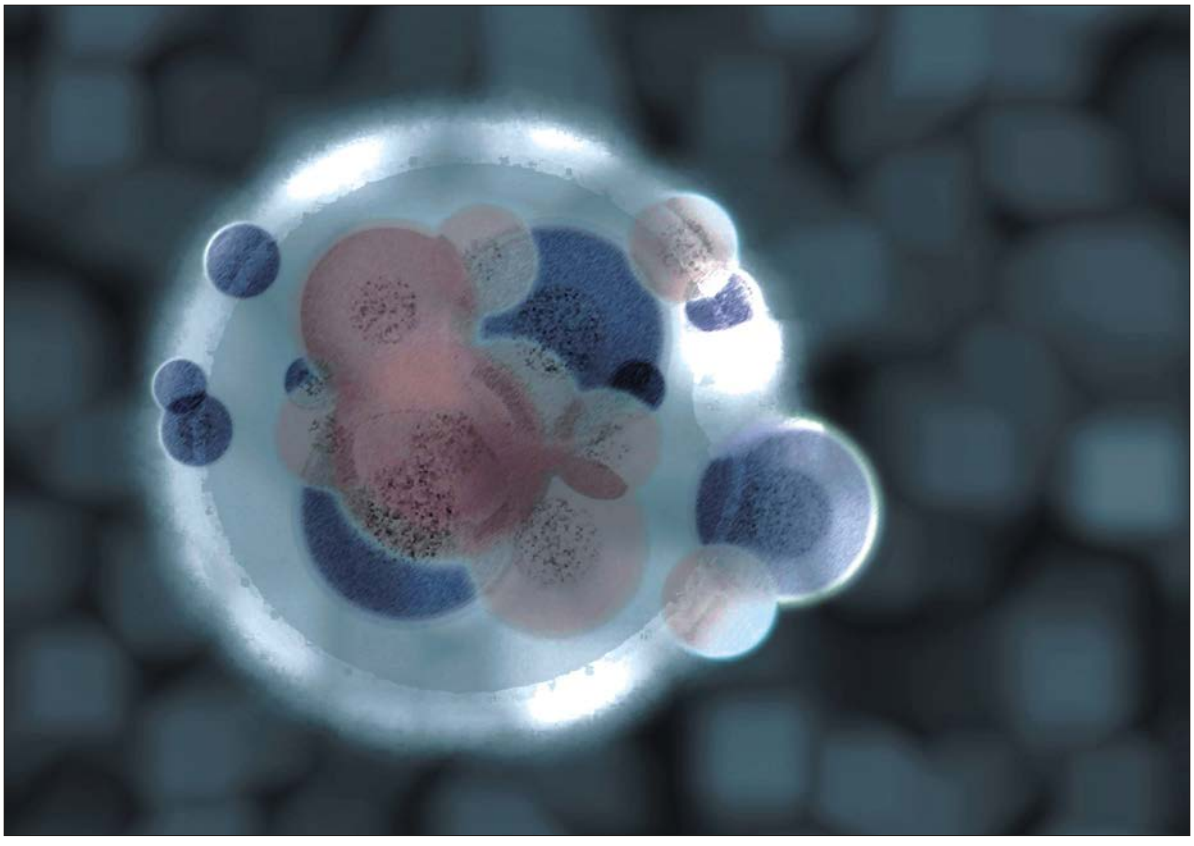

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An Exceptionally Short α-actinin-like Protein from the Protozoan Parasite *Entamoeba histolytica*

[1]Heike Bruhn, [2]Thomas Jacobs, [3]Britta Urban and [4]Matthias Leippe
[1]Research Center for Infectious Diseases, Würzburg, Germany,
[2]Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany,
[3]Institute for Molecular Medicine, John Radcliffe Hospital, Oxford, UK,
[4]Zoological Institute, University of Kiel, Germany

Abstract: For a primitive phagocytosing cell such as the protozoan *Entamoeba histolytica*, dynamic fluctuations of the cytoskeleton are crucial for morphological changes involved in cellular movement as well as in phagocytosis. Both activities are implicated in the virulence of the human pathogen. Here, this study molecularly cloned an amoebic α-actinin, one of the components essential for actin bundling. The isolated cDNA codes for a protein of 537 amino acid residues, which displays two calponin homology (CH) domains and three EF-hand motifs. Although obviously belonging to the α-actinin-family, the protein has several features unusual for an α-actinin concerning its domain architecture. Structurally, this protein is the shortest member of the α-actinin family known so far.

Key words: *Entamoeba histolytica*, α-actinin, actin-binding protein, cytoskeleton, EF-hand

INTRODUCTION

Human amoebiasis is caused by the protozoan *Entamoeba histolytica*, a parasite, which resides and proliferates in the colon of its host. The pathogenesis of the invasive form of the disease involves the dissemination of the amoebae from the large bowel and their invasion into human tissue, mostly resulting in severe liver abscesses. The amoebae actively move from the lumen into the intestinal mucosa and may enter the blood stream. Finally, they may reach extraintestinal tissues via the circulation. Concomitantly, killing and phagocytosis of host cells result in marked tissue destructions. Besides its medical importance, *E. histolytica* appealed to biologists of various disciplines because of its unusual simplicity in subcellular structures, its biochemical peculiarities and its enormous phagocytic and cytolytic potential. Two cellular processes, active locomotion as well as phagocytosis are vital for the parasite and are also crucial for its pathogenic behaviour.

As both, locomotion and phagocytosis, are driven by the cytoskeleton, much interest has been focused on characterization of proteins involved in cytoskeletal rearrangement and actin binding[1]. Several actin-binding proteins of *E. histolytica* have been characterized at the molecular level or identified by immunological cross-reaction[2-7]. The organization of individual actin filaments into higher ordered structures is controlled by actin-crosslinking proteins. The largest group of these proteins is characterized by a conserved actin-binding domain and comprises α-actinin, fimbrin, dystrophin, spectrin and parvin[8,9]. Here, the identification and characterization of an α-actinin-like protein of *E. histolytica,* is reported which presents some unique features. This α-actinin-like protein is the shortest one known so far, both in sequence and in structure. α-actinins are ubiquitous proteins involved in the bundling of actin filaments; they are homodimeric proteins containing two actin-binding domains – one at the N terminus of each monomer - which are separated by a long rod consisting of four spectrin repeats per monomer[10]. The monomers are orientated in an antiparallel fashion using the spectrin repeats as a dimerization interface. Each of the actin-binding sites can bind one actin filament resulting in their cross-linking. The C terminus of α-actinins usually contains two calcium-binding EF-hand motifs, which interact with the actin-binding domain of the other monomer and – in the case of nonmuscle α-actinin – regulate the actin-binding capability. In recent years, multiple cellular functions of α-actinins were discovered beyond the mere actin-bundling function and the list of putative interaction partners is still growing[11]. Accordingly, mammalian α-actinins link the cytoskeleton to a variety of

Corresponding Author: Dr. Matthias Leippe, Zoological Institute, University of Kiel, Olshausenstr. 40, 24098 Kiel, Germany
Tel: +49-431-880-4196 Fax: +49-431-880-4197 E-mail: mleippe@zoologie.uni-kiel.de

transmembrane proteins, regulate the activity of several receptors and may be involved in signal transduction pathways. A likewise important function of the here described novel actinin-like protein of *E. histolytica* might be assumed.

MATERIALS AND METHODS

Molecular cloning: The oligonucleotide 5´AA$^{A}/_{G}$TT$^{T}/_{C}$CAATGGTGGTT$^{T}/_{C}$CC and an Oligo-dT(18) primer were used to amplify a cDNA fragment from 1x10^{6} pfu of a λ ZAP cDNA library of the *E. histolytica* strain HM1:IMSS . The resulting fragment was used as a probe for the screening of the same library. The sequences of three positive clones were identified by standard sequencing methods. The longest sequence was deposited at the EMBL/GenBank/DDBJ databases (acc: AF208390).

Sequence characterization: Homologues sequences were identified by blast-search (http://www.ncbi.nlm.nih.gov/BLAST/) and by motif characterization using InterPro (http://www.ebi.ac.uk/interpro/). Sequence alignments were performed by Clustal W[12] and coloured for presentation with Macboxshade (http://www.isrec.isb-sib.ch/ftp-server/boxshade/). Sequence identities were calculated by Blast-two-sequences ((http://www.ncbi.nlm.nih.gov/BLAST/),). Coiled coils were predicted by the programs COILS[13] and Paircoil[14], the differentiation between two- and three-stranded coiled coils were performed using Multicoil[15]. The three dimensional structure of the spacer region was modeled using SwissPDB-Viewer[16].

RESULTS AND DISCUSSION

Molecular cloning: While searching for the calcium-binding proteins named grainins of *E. histolytica*[17], we identified another putative calcium-binding protein. A 258 bp DNA fragment amplified using a λzap cDNA library as a template was used to screen the same library. The DNA sequence of the longest of three positive clones yielded an open reading frame of 1614 bp, which is in good agreement with a signal on a Northern Blot of ~1.7 kb (data not shown). The deduced amino acid sequence appears to be complete and reveals a protein of 537 amino acids residues.

Primary structure and domain organization: It is hypothesized that this protein is an α-actinin-like protein because i) it possesses 2 CH domains and EF-hand motifs. Although certainly several actin-binding proteins possess CH domains and EF-hands for the binding of calciumons are found in a variety of proteins, the combination of both structural properties in this order is truly characteristic for α-actinins or their larger relatives of the same actin-crosslinking family. ii) it possesses a spacer region between the actin binding and the EF-hand domain. Interestingly, in the *E. histolytica* protein, these domains are separated by a helical structure of only 90 amino acid residues. Correspondingly, whereas typical α-actinins are ~100 kDa proteins[8], the calculated molecular mass of the amoeba protein is with 63 kDa substantially lower. All other known proteins consisting of both mentioned components, the twofold CH domains and EF-hand domains, i.e. dystonin or microtubule-actin crosslinking factor, possess an even more extended spacer region and, notably, additional functional domains, suggesting that it is reasonable to classify the novel protozoan protein as a member of the α-actinin-family. The only other α-actinin-like protein with unusual spacer region has been found in *Trichomonas vaginalis*; its separating sequence comprises five repeats of about 100 amino acid residues each with a yet unknown structure.

In α-actinins, the two CH-domains mediate the binding of actin; their name refer to the muscle regulating protein calponin[10,18]. The combination of two CH-domains is a common motif also in other actin-binding proteins such as spectrin, fimbrin, dystrophin, actin-binding protein 120 (ABP120), cortexilin and parvin. Only the first CH-domain appears to be actually required for binding of actin, whereas the second, C-terminal domain enhances the binding affinity but does not bind actin by itself alone[19]. The identity of the actin-binding region of *E. histolytica* α-actinin-like protein to those of other actin-binding proteins is about 30%. Notably, most of the known sequences are from metazoa. In an alignment of the actin binding regions of α-actinins, a sequence divergence between proteins of protozoan and metazoan origin becomes apparent, which may reflect the evolutionary distance between the unicellular organisms and higher eucaryotes (Fig. 1). However, the sequence identity of the *E. histolytica* protein domains to the α-actinin of the putatively closer related amoeboid protozoan *D. discoideum* is also 28% only. The amino acid residues required for actin-binding are not clearly mapped within the actin-binding domains, but can be located to a hydrophobic surface groove around a helical stretch comprising the C terminus of the first CH-domain[19]. Particular residues as well as the overall hydrophobic character of this patch are well conserved in the *E. histolytica* protein (Fig. 1). The structural membership of the amoeba protein to the α-actinin family is also indicated by the conservation of amino acid residues providing the hydrophobic protein core within the CH-domains.

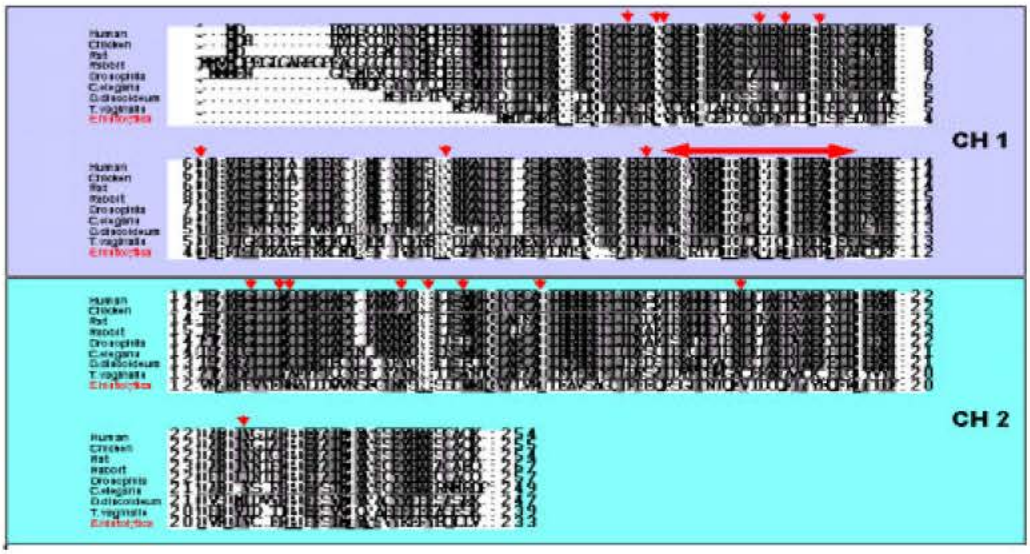

Fig. 1: Sequence alignment of the actin-binding regions of representatives of known α-actinins and of the *E. histolytica* α-actinin-like protein performed by Clustal[29]. The CH domains are shown separately. The accession numbers of the various α-actinins are: human (AAA51582.1), chicken (AAA48570.1), rat (AAC53102.1), rabbit (S17548), drosophila (FAFFAA), *C. elegans* (A48403), *D. discoideum* (FADOAA), *T. vaginalis* (AAC72899.1) and *E. histolytica* (AF208390). The putative actin-binding region is marked by a bold horizontal arrow and hydrophobic residues necessary for protein core interactions in known structures are marked by small vertical arrows. Identical amino acids are presented in white colour on a black background, conserved residues (>60% identity in between the sequences) are shaded darkly grey and amino acid residues with similar properties are shaded lightly grey

In all known α-actinins, the C-terminal domain of a monomer consists of two EF-hands, but only the actin-binding activity of nonmuscle α-actinins is reported to be regulated by calcium[8]. In the antiparallel α-actinin dimer, the EF-hand domain of one monomer interacts with the actin-binding domain of the other. The only exception from the rule is the α-actinin of *T. vaginalis* in that it contains a sole functional EF-hand motif[20]. Whether the activity of this protein is regulated by calcium is not reported so far. The *E. histolytica* α-actinin-like protein described here comprises three functional EF-hand motifs which reveal the highest sequence identity to the respective motifs of the recently identified grainins, calcium-binding proteins from cytoplasmic granules of *E. histolytica*[17]. In most of the sequences with an odd number of such motifs, an additional ancestral one can be identified. Accordingly, the secondary structure prediction for the *E. histolytica* α-actinin-like protein suggests a fourth helix-loop-helix motif. As found with the grainins, the overall shape of the C-terminal domain comprising the three EF-hands and the additional ancestral EF-hand is compact and globular in contrast to other dumbbell-shaped calcium-binding proteins, e.g. calmodulin, due to the short sequence distance between the second and third EF-hand motif.

With regard to the spacing segment between the actin-binding sites of the α-actinin dimer, this region comprises in the *E. histolytica* protein an helical stretch with an overall length of about 90 amino acid residues. The composition of the helix is given by repeats of a highly charged eight amino acid motif (A/Q K/R E E Q E R L/I/K). It resembles the sequence of the straight central helix of caldesmon[21], which also separates two independent globular domains. The unusual high charge density is able to stabilize the helical structure by an extraordinary number of interhelical electrostatic interactions involving the residues in position i and i+4[22]. In the *E. histolytica* α-actinin-like protein, 24 salt bridges can be formed leaving only seven glutamate residues without binding partner (Fig. 2). Assuming a twist as typical for interacting helices, these negative charges are located on one face of the helix only, providing the other, unpolar side as a dimerization-scaffold for a coiled coil structure. The propensity to form a coiled coil is strongly predicted by two independent prediction programs[13,14].

If a two-stranded antiparallel coiled coil built by two monomers is assumed for the amoeba protein, the length of this separating rod is approximately 120 Å which is in clear contrast to the 240 Å rod of typical α-actinins formed by four spectrin repeats[23] (Fig. 3). In an α-actinin

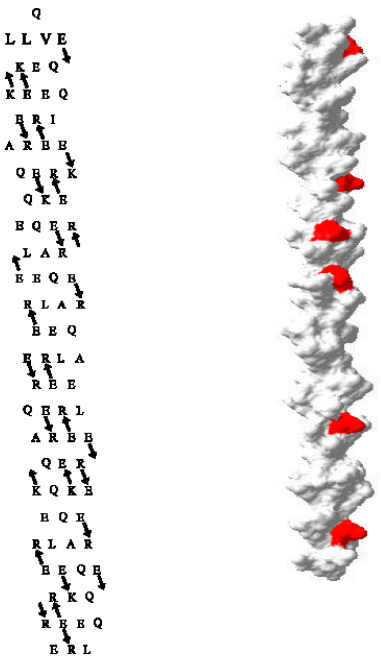

Fig. 2: Helical spacer region of the *E. histolytica* α-actinin-like protein. Potential interhelical electrostatic interactions are shown by a helical-net presentation (left). The helix is cut lengthwise and spread out two-dimensionally. The entire amino acid sequence of the region is given in the one letter code and putative salt bridges are indicated by arrows. The sole weaker i, i+3 interaction is transparently shown. The molecular surface of this region was modeled using SwissPDB-Viewer (right). The one-sided distribution of the free glutamate residues not involved in salt bridges (red) becomes evident.

monomer, each single spectrin repeat is folded into a three-stranded antiparallel coiled coil, which assembles with that of the other monomer to a dimeric, very rigid structure. According to a prediction program which differentiates between trimeric and dimeric coiled coils[15], such a trimeric antiparallel coiled coil cannot be built by the 90-residues repeat domain of the amoebic α-actinin-like protein and consequently a short version of a typical α-actinin-rod is not likely to exist in that protein.

The distance of the two actin-binding sites of actin-bundling proteins determines the type of the actin structures resulting from the cross-linking process. The adjacent sites in the 14-nm fimbrin molecule, for example, result in tight bundling, whereas α-actinin molecules of typically 40 nm direct the formation of more loose assemblies thereby providing space for the interaction of actin filaments with other proteins, e.g. myosin thick filaments[24]. The intermediate cross-linking distance provided by the *E. histolytica* α-actinin-like protein may therefore result in a distinct interaction pattern of the actin fibers. α-Actinin is localized in focal adhesion plaques, which anchor the actin filament to the membrane and are the initiation site of signal transduction events such as the protein kinase C and adenylate cyclase pathways in response to cell adhesion. Accordingly, an α-actinin-like protein of *E. histolytica* was localized by immunological cross reaction with to actin-rich structures[3], which resemble the adhesion plaques of higher eucaryotes[2]. Within these cellular structures of *E. histolytica*, the host extracellular matrix protein fibronectin and its proteolytic degradation fragments trigger signaling pathways resulting in reorganization of the cytoskeleton to facilitate locomotion and chemotaxis[25,26]. It has been proposed that such specific signaling pathways could be involved in and promote the invasive behaviour of the pathogen. In

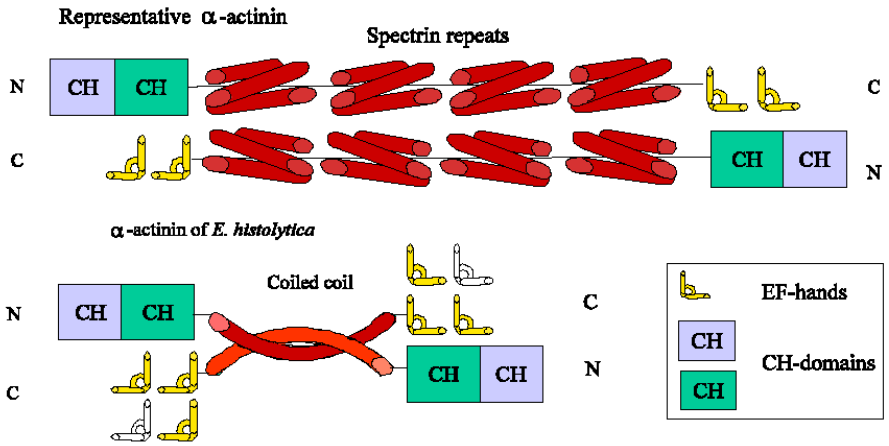

Fig. 3: Domain architecture of a representative α-actinin in comparison to the α-actinin-like protein of *E. histolytica*. In the EF-hand region, the fourth helix-loop-helix motif of the *E. histolytica* α-actinin-like protein is not shaded because it is considered non-functional

adhesion plaques, α-actinin usually interacts with several different proteins. The binding site of many of them is mapped to the spectrin-repeat rod not existent in the *E. histolytica* α-actinin-like protein[23,27]. With regard to the separation structure of the actin-binding sites, the *T. vaginalis* α-actinin is also unusual in that it comprises a yet unknown structure built by five repeats of about 100 residues[20]. The protein is colocalized with actin particularly in pseudopods of the amoeboid form of this flagellate, but other interaction partners are not defined so far. The surface of the spectrin-repeat rod of typical α-actinins is predominantly negatively charged to enable the interaction with the mainly basic cytoplasmic tails of transmembrane proteins[28]. Notably, this feature is also prevalent in the helical spacer of the *E. histolytica* α-actinin-like protein, tempting to speculate, that the binding properties of the potential partners are also suited for this divergent structure. Alternatively, this unusual protein might differ in interaction partners and accordingly in function from the known α-actinins.

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